Maturation of Borna disease virus glycoprotein

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Received 4 May 2005; revised 28 June 2005; accepted 19 July 2005

Available online 11 August 2005

Edited by Shou-Wei Ding

Abstract The maturation of Borna disease virus (BDV) glycoprotein GP was studied in regard to intracellular compartmentalization, compartmentalization signal-domains, proteolytic processing, and packaging into virus particles. Our data show that BDV-GP is (i) predominantly located in the endoplasmic reticulum (ER), (ii) partially exists in the ER already as cleaved subunits GP-N and GP-C, (iii) is directed to the ER/cis-Golgi region by its transmembrane and/or cytoplasmic domains in CD8-BDV-GP hybrid constructs and (iv) is incorporated in the virus particles as authentic BDV glycoprotein exclusively in the cleaved form decorated with N-glycans of the complex type. Downregulation of BDV-glycoproteins on the cell surface, their limited proteolytic processing, and protection of antigenic epitopes on the viral glycoproteins by host-identical N-glycans are different strategies for persistent virus infections.

Keywords: Glycoprotein cleavage; Endoplasmic reticulum-localization; Transport retardation

1. Introduction

Viral glycoproteins are integrated in viral envelopes as spikes. They bind to host cell receptors before they mediate fusion between virus envelope and cellular membranes. This mechanism allows the delivery of nucleocapsids into the cytoplasm of cells and initiates virus replication. Moreover, viral glycoproteins are also main targets for the immune defense. Downregulation of viral glycoproteins on the cell surface, their limited proteolytic processing, and protection of antigenic epitopes on the viral glycoproteins by host-identical N-glycans are different strategies of virus escape from antiviral host controls. These mechanisms are extremely important for persistent virus infections and are suspected to be used also in persistent infections with the neurotropic Borna disease virus (BDV).

BDV is the causative agent of Borna disease (BD), a neurological disorder based on a T cell-mediated immunopathological reaction. BD occurs in horses and other warm-blooded animals; it is also suspected to cause psychiatric disorders in man, although this matter has not yet been definitely clarified [1,2].

BDV is the only representative of the *Bornaviridae* family. The BDV fine structure reveals a spherical enveloped virus particle with 90–130 nm in diameter covered by glycoprotein spikes [3]. BDV contains a single negative stranded, non-segmented RNA genome of 8.9 kb, which characteristically replicates and transcribes in nuclei of infected cells [4]. The BDV genome comprises six open reading frames, encoding the nucleoprotein NP, the phosphoprotein P, the protein p10 or X, and the RNA depending RNA polymerase L as components of the ribonucleoprotein complex and two membrane proteins, the lipid-associated matrix protein M [5] and the membrane-integrated glycoprotein GP [6–8].

BDV-GP is the only glycoprotein of BDV, which is also designed as gp84, gp94 or GP [9–11]. It is synthesized as a 57 kDa polypeptide precursor molecule which is translocated across the endoplasmic reticulum (ER) membrane and inserted in the ER membrane as a type I membrane protein. In the lumen of the ER, GP is co-translationally N-glycosylated and reaches a molecular mass of about 94 kDa. GP is needed to be proteolytically processed to gain fusion capacity of GP and infectivity of BDV [11]. GP is cleaved C-terminally at a tetra-basic motif RRRR by furin or by a closely related subtilisin-like protease into two subunits, the distal N-terminal GP-N and the C-terminal, membrane anchored GP-C. GP-N is sufficient for receptor binding and GP-C mediates fusion between viral envelope and host membranes [12,13]. Subcellular localization, transport, and incorporation of the BDV-GP into virions are not well understood events. Therefore, it was the aim of this study to identify the intracellular localization of non-cleaved precursor GP and cleaved GP-N/GP-C complex, and to determine the strategy used for the downregulation of precursor BDV-GP transport.

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Abbreviations: aa, amino acid; BDV, Borna disease virus; BDV-GP, Borna disease virus glycoprotein; BIP, immunoglobulin heavy chain binding protein; ConA, concanavalin A; CT, cytoplasmic tail; Endo H, endoglycosidase H; ER, endoplasmic reticulum; GP, glycoprotein precursor (gp84/gp94); GP-C, carboxy-terminal glycoprotein subunit (gp43); GP-N, amino-terminal glycoprotein subunit (gp51); GNA, *Galanthus nivalis* agglutinin; HRP, horse radish peroxidase; PNGase F, peptide-*N*-glycosyidase F; Rb-αGP-C, rabbit antiserum against carboxy-terminal BDV-glycoprotein; Rb-αGP-N, rabbit antiserum against amino-terminal BDV-glycoprotein; sulfo-NHS-biotin, sulfo-*N*-hydroxysuccinimide biotin; TM, transmembrane domain; Vero cells, African green monkey kidney cells; WGA, wheat germ agglutinin

0014-5793/$30.00
doi:10.1016/j.febslet.2005.07.052

2. Materials and methods

2.1. Cell culture and purified virus preparation

COS-7-cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (v/v), 50 units/mL penicillin, 50 μg/mL streptomycin, and 2 mM L-glutamine. All media and cell culture reagents were purchased from GIBCO™ Invitrogen Inc. (Karlsruhe, Germany).

BDV particles were prepared from about 3×10^8 permanently BDV-infected confluent grown MDCK-cells. For this purpose, BDV-infected cells were washed two times with 20 mM HEPES-buffer (pH 7.4), incubated for 1.5 h at 37 °C with 20 mM HEPES-buffer (pH 7.4) supplemented with 250 mM $MgCl_2$ and 1% fetal calf serum (v/v) [14]. The supernatants of magnesium chloride-treated, BDV-infected cells were collected, cleared from cell debris by centrifugation at $2500 \times g$. Then the viral particles were spun down for 1 h through a 6 mL sucrose cushion at $80\,000 \times g$ (SW28 rotor, ultracentrifuge, Beckmann). The virus fraction was resuspended in 1 mL PBS, loaded onto an equidistant 12-step Optiprep-gradient, ranging from 12% to 30% Iodixanol in PBS (Sigma Deisenhofen, Germany). The gradient was centrifuged at $250\,000 \times g$ for 2 h (SW41 rotor, Beckmann). Aliquots (100 μL) of each gradient fraction (0.5 mL) were analyzed by infecting confluent MDCK cell cultures grown in 96-well plates. After 1 h, the inoculums of the cells were replaced by Dulbecco's medium containing 2% fetal calf serum. The cells were further incubated for 4 days at 37 °C, before the numbers of BDV-infected cells were determined by fluorescence forming units per mL (FFU/mL) [11].

2.2. Lectin precipitation, endoglycosidase treatment of BDV-glycoprotein

A purified BDV preparation (100 μL) was solubilized in 900 μl GDK-1 buffer [15] and centrifuged 30 min at 13 000 rpm. The supernatant was incubated with 50 μL lectin-Sepharose, as indicated in Fig. 4, for 16 h at 4 °C. The precipitated samples were twice washed with 1 mL of GDK-1 buffer. Purified BDV-GP was either directly subjected to SDS–PAGE and electrophoretic transferred onto nitrocellulose membranes (NC) or PNGase F treated before SDS–PAGE. The NC-blot was treated with PNGase F for 16 h at room temperature as described before [15] and different BDV glycoprotein forms detected by immunoblot analysis (see Section 2.3).

2.3. Subcellular fractionation, SDS–PAGE, and immunoblot analysis

Fractionation of subcellular compartments was performed as formerly described [16]. About 10^7 C6-cells permanently infected with BDV strain He80 were cultured to 100% confluence, washed two times with buffer A containing 0.25 M sucrose, 10 mM triethanolamine, 1 mM EDTA, pH 7.4, scrapped in 5 mL homogenization buffer containing 85% buffer A (v/v) and 15% of buffer B (10 mM Tris, pH 7.4, 5 mM KCl, 1 mM EDTA, and 128 mM NaCl) and centrifuged at $300 \times g$ for 10 min at 4 °C. Cells were resuspended in 300 μL of homogenization buffer and homogenized at 4 °C by 12 times passages through a 25-gauge needle attached to a 1-ml syringe. Fragmented cells were precipitated at $1000 \times g$ for 10 min at 4 °C and the homogenization procedure was repeated once. The combined post-nuclear supernatants were loaded on a linear 0–26% Optiprep gradient in buffer B and centrifuged for 2 h at 41 000 rpm at 4 °C in a SW41 rotor. Each of the 700 μL fractions were diluted with 700 μL buffer B and the fragmented cellular membranes were precipitated at $125\,000 \times g$ (TLA45 rotor, Beckman High Speed Microfuge) for 60 min at 4 °C. Each pelleted fraction was dissolved in 50 μL SDS–PAGE sample buffer. The proteins were separated by SDS–PAGE and analysed by immunochemical reaction after transfer onto nitrocellulose membranes. For this, the membranes were incubated for 1 h with one of the following primary antibodies which were diluted in PBS containing 0.1% Tween 20, BDV-GP-N or respective BDV-GP-C specific polyclonal anti-peptide sera from rabbits (Rb-αGP-N and RB-αGP-C)[15], GM130 specific monoclonal antibody, BIP-specific monoclonal antibody (Transduction Laboratories, Lexington, USA), followed by an incubation with horse radish peroxidase (HRP)-conjugated mouse or rabbit IgG specific secondary antibody (DAKO, Hamburg, Germany), respectively. Immunoreactive HRP-tagged protein bands were visualized by chemiluminescence using Super Signal substrate (Pierce, Rockfort, USA).

2.4. Chimeric protein constructs of CD8 α-chain and BDV-glycoprotein domains

The fusion proteins CD8-BDV-CT, CD8-BDV-TM and CD8-BDV-TM/CT were generated by subcloning of the cytoplasmic tail (aa 486-503, CT) and of the transmembrane/cytoplasmic BDV-GP domains (TM aa 463-485, TM/CT aa 463-503) (Fig. 3A). The encoding cDNA sequences of TM/CT and CT were amplified by using RT-PCR and viral (v) RNA isolated from Vero cells infected with BDV strain No98 [17] and an RNeasy Mini Kit as described by the supplier (Qiagen, Hilden, Germany). To obtain the desired CD8-BDV-TM DNA construct, synthetic complementary oligonucleotides comprising TM were amplified via PCR to supplement double stranded DNA fragments. The TM domain was inserted into the cloned CD8 gene after digestions with corresponding restriction endonucleases. Following restriction sites, *Xba*I and *Bam*HI (TM/CT), or *Eco*RI and *Bam*HI (CT), or *Xba*I and *Bam*HII of the plasmid pCMUIV-CD8 were used for the insertion of BDV-GP specific cDNA fragments. The modified pCMUIV-CD8/E3-K19 was used as ER-control [18].

2.5. Transfection, eukaryotic expression and surface biotinylation

Overnight COS-7 cell cultures (3.5 cm dishes) were transfected with 1 μg of plasmid as indicated using Lipofectamine transfection reagent (Invitrogen, Karlsruhe, Germany). At 48 h post-transfection, the cells were fixed and subjected to indirect immune fluorescence staining. The plasmid pECFP encoding an enhanced cyan fluorescent protein fused to a fragment of the β-1,4-galactosyltransferase was used as a marker for trans-medial region of the Golgi apparatus (BD Bioscience Clontech, Heidelberg, Germany). Surface biotinylation was performed as previously described [19].

2.6. Indirect immunofluorescence

Permeabilized cells were fixed with pre-cooled methanol/acetone (1:1) for 10 min and kept on ice during all subsequent incubation and PBS washing steps. Non-permeabilized cells were directly incubated with antibodies for surface proteins/markers. Cells were incubated for 1 h with primary antibodies. The second incubation was for 1 h with the corresponding secondary antibodies or reagents.

3. Results

3.1. Subcellular localization of BD-GP in virus-infected cells

BDV-infected cells clearly show that BDV-GP is localized dominantly in subcellular compartments, most likely the ER and/or Golgi region, after membrane permeabilization [12]. The transport to the plasma membrane is not very efficient, since only weak fluorescence signals are detectable on the cell surface of non-permeabilizied, BDV-infected cells (Fig. 1). These results suggest an inefficient transport of BDV-GP to the cell surface.

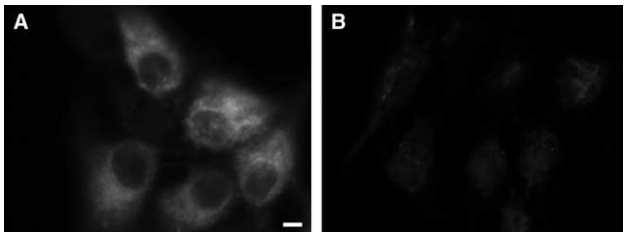

Fig. 1. Inefficient transport of BDV-GP to the cell surface. BDV-infected BHK-T7 cells were first fixed by acetone/methanol and then incubated with antiserum Rb-αGP-C and the BDV-GP was immunostained by rabbit-IgG conjugated to fluorescein (A), or cells were incubated with Rb-αGP-C first, subsequently and fixed. The BDV-GP was stained with rabbit-IgG conjugated to Alexa Fluor®594 (B). Scale bar represents 10 μm.

3.2. Subcellular distribution of non-cleaved and cleaved BDV-GP

In order to investigate the intracellular distribution of non-cleaved and cleaved glycoprotein, permanently BDV-infected C6-cells were homogenized and the subcellular compartments were fractionated by density-gradient ultracentrifugation. Each gradient fraction was subjected to SDS–PAGE and immunoblotting. Non-cleaved GP and GP-C of the GP-N/GP-C complex were detected on blot membranes by a GP-C specific antiserum (Fig. 2A). The cellular compartments of interest, the ER and the Golgi apparatus, were identified by antibodies raised against the ER-located chaperone BiP (Fig. 2B) and the cis-Golgi matrix protein GM130 (Fig. 2C), respectively. As shown in Fig. 2, both forms of the glycoprotein, non-cleaved GP and cleaved GP-N/GP-C-complexes, are predominantly localized in the fractions containing ER-specific proteins and not in fractions containing the Golgi region. BDV-GP-N-specific serum revealed similar results (data not shown). The detection of BDV-GP in the fractions containing ER-specific proteins is comparable with data obtained by Gonzalez-Dunia and co-workers [9]. Investigations on the processing of GP oligosaccharide side chains by treatment with endoglycosidases show that BDV-GP and GP-C retain Endo H sensitive.

3.3. Determinants of subcellular transport and localization of the BDV-GP

We were interested in BDV-GP domains which determine the intracellular distribution of the viral glycoprotein. It is known from quite a number of integral membrane glycoproteins that their localization signals reside in either the cytoplasmic or the transmembrane region or both. In order to investigate the glycoprotein domains for localization signals, we constructed protein hybrids consisting of the cytoplasmic and/or transmembrane domains of BDV-GP with various domains of the CD8 molecule (Fig. 3A). CD8 is a type I membrane protein found on cytotoxic T-cells, which has been previously used as a reporter molecule; as a wild type molecule, it is efficiently transported to the plasma membrane (Fig. 3B, a, f, k). As a control for ER localization, we used a truncated CD8 protein fused to an ER-retention signal peptide taken from the adenoviral E3/K19 protein (Fig. 3B, b, g, I). Transfected and permeabilized COS-7 cells were stained with the anti-CD8 monoclonal antibody to demonstrate the intracellular expression of all chimera constructs (Fig. 3B, a–e). In non-permeabilized, transfected COS-7 cells only chimeric proteins are detected which are expressed on the cell surface (Fig. 3B, f–j). Besides the wild type CD8 control, only the chimeric protein CD8-BDV-CT was detectable on the cell surface (Fig. 3B, i). The two other constructs CD8-BDV-TM/CT and CD8-BDV-TM as well as the ER-control CD8-E3/K19 were not transported to the cell surface (Fig. 3B, g, h and j). Double staining of the non-permeabilized cells with a GM1-specific antiserum was used to demonstrate the validity of the cell surface staining (Fig. 3B, k–o). In order to confirm these results by another independent method, COS-7 cells were transfected with the same constructs as shown in Fig. 3. In this experiment, cell surface proteins were labeled by sulfo-NHS-biotin, which does not penetrate into the cytoplasma of cell. Using this method, again only the CD8-BDV-CT and CD8 proteins were labeled with biotin after precipitation with a CD8 specific antibody and Western blot detection using streptavidin-peroxidase staining (Fig. 3C). Taken together, our observations indicate that the transmembrane domain of BDV-GP possesses a retention or retrieval signal which directs the CD8-BDV-TM and CD8-BDV-TM/CT proteins in an early cell compartment, most likely in the ER or cis-Golgi and does not allow cell surface transport. Investigations to identify ER-retention signals within the ektodomain of BDV-GP are in progress. However, BDV-GP expression of mutated GP-forms which were used is inefficient for this analysis (data not shown).

In order to precisely analyze the compartment(s) where BDV-GP mostly resides, the CD8-chimeric constructs were cotransfected with a β-1,4-galactosyltransferase fragment, a marker for the trans-medial Golgi region (Fig. 3D). The CD8-BDV-TM and CD8-BDV-CT chimera are transported from the ER to the Golgi-cisternae as indicated by co-localisation of the Golgi- and CD8-markers (yellow; Fig. 3D, g and h). Interestingly, the CD8-BDV-TM/CT chimeric protein did not show co-localization of CD8 and Golgi markers (Fig. 3D, i). These data clearly indicate that the information for strict ER-localization needs both, the transmembrane and cytoplasmic domains of BDV.

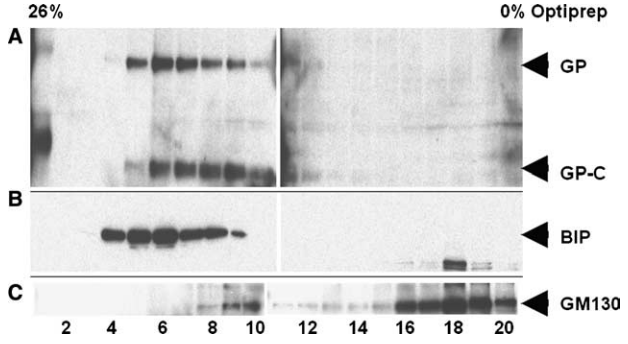

Fig. 2. Subcellular Distribution of BDV-GP and its mature form GP-N/GP-C. Permanently BDV-infected C6 cells were lysed and the post-nuclear supernatants were subjected to velocity gradient centrifugation containing 0–26% iodixanol. Aliquots of the collected gradient fractions were analyzed on immunoblots after SDS–PAGE using Rb-αGP-C (A), anti-BiP as ER-marker (B), and antibodies directed against matrix protein GM130 as cis-Golgi marker (C).

3.4. Selective incorporation of only cleaved BDV-GP in virus particles

Until now, it was known that full length GP and GP-C are presented on infectious virus particles [9]. Weather the N-terminal cleavage fragment GP-N is located on the BDV particle is still unclear. Therefore, highly purified BDV particles were prepared from BDV-infected MDCK-cells. The virus release was induced by a salt shock and virus particles were purified by density gradient ultracentrifugation. The GP-C specific antiserum recognizes only one main band with an apparent molecular mass of 43 kDa representing the membrane anchored subunit GP-C when Concanavalin A was used for the precipitation (Fig. 4A, lane 1). *Galanthus nivalis* agglutinin (GNA) and wheat germ agglutinin (WGA) were also able to precipitate GP-C (Fig. 4A, lanes 2 and 3). When using the GP-N-specific serum for immunodetection of lectin-precipitated BDV-GP, a series of distinct bands comprising a molecular mass range from 45 to 55 kDa was detected, indicating micro-heterogeneity of GP-N due to imperfect N-glycosylation

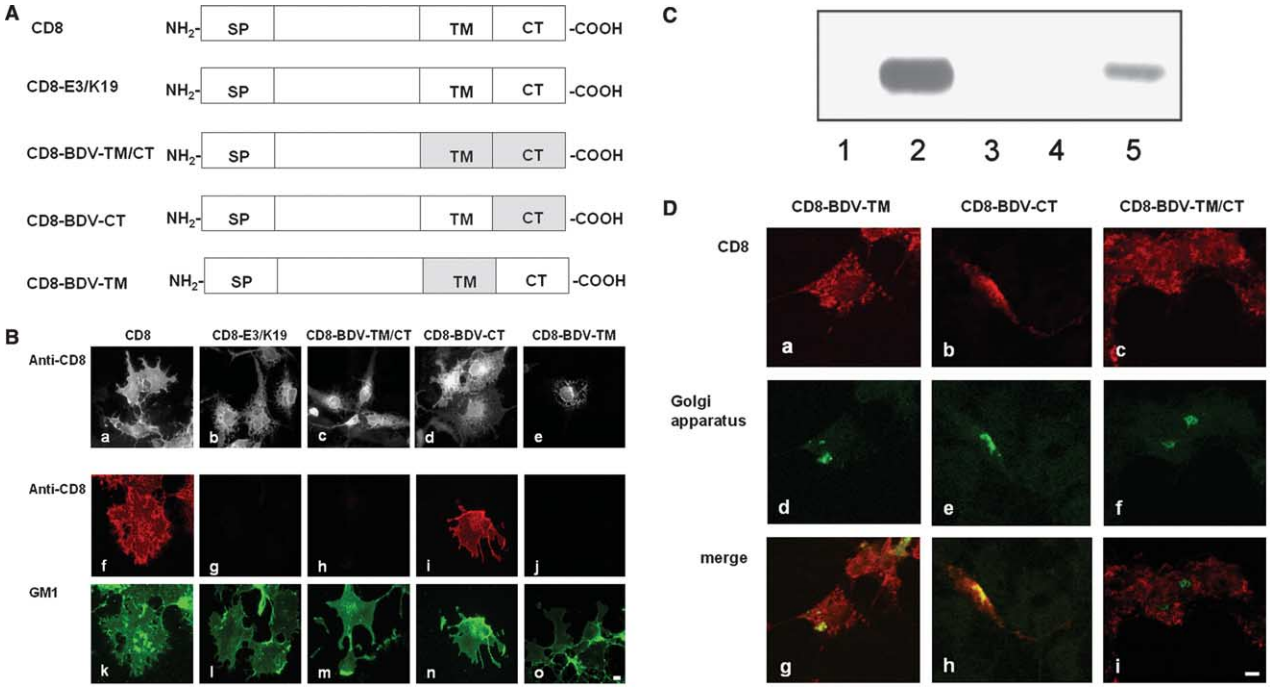

Fig. 3. BDV-glycoprotein domains responsible for ER/cis-Golgi residence. (A) Schematic presentation of CD8-BDV-GP chimeric proteins and CD8-E3/K19-chimeric ER-resident marker protein which were used for transfection of COS-7 cells. BDV-GP specific domains are symbolized as grey rectangles. SP, signal-peptide; TM, transmembrane domain; CT, cytoplasmic domain. (B) COS-7-cells were transfected with the indicated chimeric constructs, after 48 h fixed with pre-cooled methanol/acetone, incubated with CD8-specific monoclonal antibody Okt8 and subsequently stained with fluorescein conjugated IgG against mouse (panels a–e). COS-7 cells transfected in parallel were first incubated with Okt8 and Alexa Fluor®594 anti-mouse IgG immunoglobulin for surface immunostaining (panels f–j) or cell surface control staining with the B-subunit of cholera toxin conjugated with biotin which reacted with streptavidin conjugated with fluorescein (panels k–o). Specimens were visualized using a Zeiss fluorescence microscope Axioplan equipped with an automatic camera system. Scale bar represents 10 μm. (C) COS-7 cells were transiently transfected with CD8-chimeras (CD8 (2), CD8-E9/K19 (3), CD8-BDV-TM/CT (4) or CD8-BDV-CT (5)). Untreated cells were used as control (1). 48 h after transfection cell surface proteins were labeled by sulfo-NHS-biotin. Cells were extracted and extracts were precipitated with CD8-specific antibodies. Precipitates were subjected to SDS–PAGE and biotinylated proteins were visualized after immunoblot using a streptavidin-peroxidase staining. (D) COS-7 cells were transiently co-transfected with indicated CD8-chimeric constructs and with the pEYFP-Golgi construct as Golgi marker protein for co-localization studies. The cells were acetone/methanol-fixed 48 hours later and stained with Alexa Fluor®594 detecting Okt8 specific for CD8 (panels a–c) or were used directly for detecting the Golgi-marker (panels d–f). Co-localization of transiently expressed proteins was depicted in yellow (panels g and h) using confocal laser scanning microscope TCS SP2 (Leica, Wetzlar, Germany). Scale bar represents 10 μm.

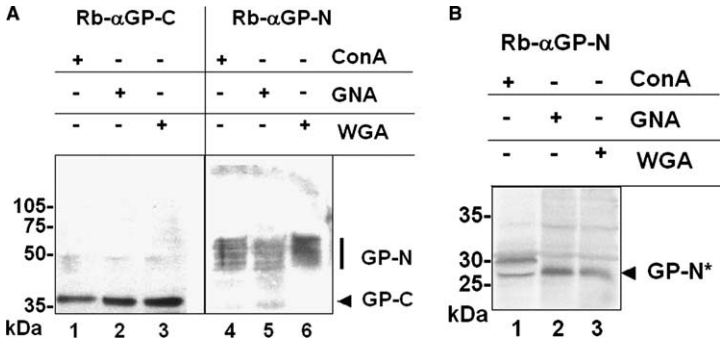

Fig. 4. Characterization of BDV-GP forms incorporated in virus particles. (A) Purified BDV particles were precipitated with indicated lectins followed first by SDS–PAGE and transfer onto nitrocellulose membrane. N-glycans were then deglycosylated by PNGase F and the deglycosylated BDV-glycoprotein detected by immunochemistry using Rb-αGP-C (lanes 1–3) or with Rb-αGP-N (lanes 4–6) and a luminescence detection kit. (B) Purified BDV particles were first precipitated by indicated lectins (lanes 1–3) and PNGase F treated prior to SDS–PAGE immunochemistry using Rb-αGP-N. GP-N* represents the completely deglycosylated form of the BDV-glycoprotein subunit GP-N. Molecular masses are indicated on the left of the panels.

(Fig. 4A, lanes 4, 5 and 6). After induction of the virus release with sodium butyrate similar results were obtained (data not shown). To further prove the detection of GP-N in virus particles, it was first precipitated by lectins (ConA, GNA, WGA), then treated with glycosidases and thereafter subjected to SDS–PAGE and blotting. The deglycosylated GP-N* was detected as a sharp band of 27 kDa (Fig. 4B, lanes 1, 2 and 3). The signal of GP-N* was partly covered by Con A (26 kDa

under reduced conditions) in lane 1. Interestingly, no non-cleaved precursor GP was detectable in the virus particles with both subunits specific antisera, although the anti-GP-C serum can recognize non-cleaved BDV-GP (cf. Fig. 2A, [15]). These findings demonstrate that subunits BDV-GP-C and BDV-GP-N, but no non-cleaved precursor GP are present in purified BDV.

4. Discussion

This is the first report which demonstrates that BDV-GP is cleaved into the GP-N/GP-C complex very early in the secretory pathway either in the ER or cis-Golgi, locations where the overwhelming amount of BDV-GP resides. Such an early proteolytic processing is unusual, but was previously documented for a misfolded pro-insulin receptor which did not leave the ER [20]. In permanently BDV infected cells a limiting portion of the GP-N/GP-C complex is delivered to the cell surface where mature BDV particles bud [3,9,15] whereas the chimera CD8-BDV-TM/CT is completely retained intracellular. Other viral proteins could be necessary for the transport of the GP-N/GP-C complex to the plasma membrane. This questions needs to be addressed.

The accumulation of BDV-GP in the ER/cis-Golgi region may be one of the possible mechanism involved in BDV-persistence. The low concentration of this viral surface protein on the cell surface may influence the efficiency of virus release, which is rather low for BDV. The main role in allowing the BDV evasion from the host immune surveillance system is the downmodulation of the functional avidity of virus-specific $CD8^+$ T-cells in BDV-induced immunopathology [21].

The transmembrane or cytoplasmic domain of BDV-GP does not contain the typical amino acid motif for ER-retention or signals for Golgi translocation or ER export. We have further examined both domains suspected to direct the subcellular transport using the CD8 alpha subunit as a suitable reporter molecule [18]. We detected a dominant retardation effect associated with the transmembrane domain, which localize TM-constructs to the ER/Golgi region of the cell. The combined TM and CT domains of BDV-GP restricted the localization of CD8-BDV-GP hybrids specifically to the ER. The suggested mechanisms may be based on interactions of the TM and CT domains with yet unknown ER-resident proteins. Length and hydrophobicity of the transmembrane domain was thought to be crucial for arresting integral membrane proteins in an early compartment [16]. However, the membrane-spanning region of BDV-GP was calculated to span 23 amino acids, enough in length to guarantee an efficient transport of BDV-GP from the ER to the plasma membrane.

We demonstrate that the subunits BDV-GP-C and BDV-GP-N, but no non-cleaved precursor GP are present in purified BDV. This observation is in contrast to previous reports [9] stating that also the full length GP is present on the BDV particle. Similar discrepancies were described for the detection of Vif in human immunodeficiency virus type virions [22]. The optiprep gradient for purification of virions represents a method to separate the majority of the non-viral extracellular molecules from infectious virion. Therefore, this method seems to be more reliable for the characterization of the protein content of the BDV particle.

Further investigations concerning the influence of correct conformation and oligomerization of BDV-GP in concert with the BDV-GP cleavage are needed to elucidate the regulation of BDV-GP export and the packaging of proteolytically processed BDV-GP into nascent virus particles.

Acknowledgments: We thank Stefan Baumeister for help with the confocal Laser microscopy. This work was supported by the *Deutsche Forschungsgemeinschaft SFB 535* and *Graduiertenkolleg* "*Proteinfunktion auf atomarer Ebene*".

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